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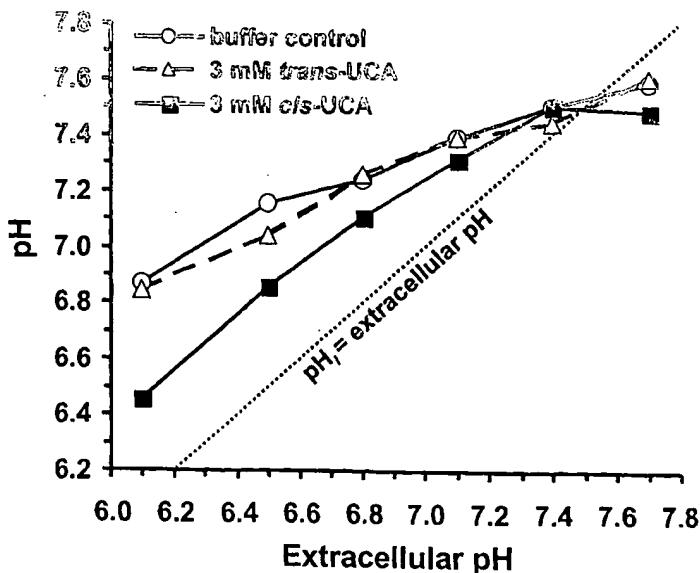
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(54) Title: PHARMACEUTICAL COMPOSITION FOR INTRACELLULAR ACIDIFICATION WITH CIS-UROCANIC



(57) Abstract: This invention relates to the use of a pharmaceutically acceptable agent being able to acidify the cell cytoplasm, for the manufacture of a pharmaceutical composition useful for causing immunosuppression in a person or an animal, wherein an effective amount of said agent is administered in an essentially non-dissociated form to the person or animal, and wherein the agent is admixed with a carrier adjusting the pH of the composition to the pH range 6.1 to 7.0. The invention also concerns a pharmaceutical composition.

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Pharmaceutical composition for intracellular acidification with *cis*-urocanic.

## FIELD OF THE INVENTION

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This invention relates to the use of a pharmaceutically acceptable agent for acidifying cell cytoplasm and subsequently causing immunosuppression in a person or an animal, and to treatment or prevention of diseases or disorders, curable by immunosuppression.

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The invention relates also to a novel pharmaceutical composition, comprising a pharmaceutically acceptable agent being able to acidify the cell cytoplasm and subsequently cause immunosuppression in the person or the animal.

## 15 BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

20

The mode of action of UV radiation in the skin is a major challenge in photoimmunology. Studies in animals and humans have established that UV exposure yields both local and systemic immunological unresponsiveness and tolerance (Schwarz 1999). The ultraviolet B (UVB) wavelengths (280–315 nm)

25 have been found to account for most of the immunosuppressive activity of UV irradiation. The agents responsible for direct absorption of the UVB photons in epidermis include urocanic acid (UCA) and DNA. Endogenous *trans*-UCA, synthesized by enzymatic deamination of histidine in the stratum corneum of the skin, is directly photoisomerized to *cis*-UCA upon exposure to UVB radiation. It  
30 has been well demonstrated, both in vitro and in vivo, that photoisomerization of UCA plays a role in UVB-induced immunosuppression. For instance, the systemic suppression induced by UVB irradiation can be largely reversed by anti-*cis*-UCA

- antibodies in mice (Moodycliffe 1996). Furthermore, in several animal models, local or systemic administration of *cis*-UCA produces immunosuppressive effects similar to UVB treatment (Gruner, 1992; el-Ghor, 1997; Garssen, 1999, Wille 1999). Some experiments have shown that UCA is capable of modulating certain 5 functions in isolated cells of the immune system *in vitro*, such as antigen presentation (Beissert 1997, Holáň 1998), NK-cell cytotoxicity (Gilmour 1993, Uksila 1994), cytokine production by spleen cells (Holáň 1998), degranulation of mast cells (Wille 1999) and activation of neutrophils (Kivistö 1996).
- 10 Neither *in vivo* nor *in vitro* studies have yet clarified which immune cells actually interact with UCA after UVB exposure and by which mechanism this molecule affects the function of the target cells at the molecular level. One would expect that UCA is a soluble mediator binding to cell surface receptors and initiating a signaling cascade. However, little is known about the putative receptor(s) of UCA.
- 15 It may share some common properties with the histaminergic system, because histamine H<sub>1</sub> and H<sub>2</sub> receptor antagonists partially block *cis*-UCA induced immunosuppression (Hart 1997). On the other hand, it has been shown that *cis*-UCA does not directly bind to histamine receptors (Laihia, 1998). Recently, displacement studies indicated that UCA may act on GABA receptors, but no direct 20 evidence of UCA binding to this receptor was demonstrated either (Laihia, 1998; Uusi-Oukari, 2000).

#### OBJECT AND SUMMARY OF THE INVENTION

- 25 The inventors of the present invention have demonstrated a so far unknown mechanism of action of *cis*-urocanic acid. They have surprisingly shown that *cis*-urocanic acid migrates into the cell cytosol in a form which is able to release a proton in the cytosol, subsequently acidify the cytoplasm, and as a result thereof, act as an immunosuppressing agent.

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Thus, according to one aspect, this invention relates to the use of a pharmaceutically acceptable agent or salt thereof being able to acidify the cell cytoplasm, for the

manufacture of a pharmaceutical composition useful for causing immunosuppression in a person or an animal, wherein an effective amount of said agent is administered in an essentially non-dissociated form to the person or animal, and wherein the agent is admixed with a carrier adjusting the pH of the composition to the pH range 6.1 to 7.0.

According to another aspect, the invention concerns a pharmaceutical composition comprising as active substance a pharmaceutically acceptable agent or salt thereof being able to acidify the cell cytoplasm, in combination with a pharmaceutically acceptable carrier, which carrier essentially prevents the agent from dissociating at extracellular pH values and wherein the carrier is able to keep the pH of the composition in the range 6.1 to 7.0.

#### BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1 is a scheme for the chemical synthesis of [<sup>14</sup>C]-radiolabeled *trans*- and *cis*-UCA.

Figure 2 shows accumulation of UCA to live neutrophils. The data points represent the mean ±SEM in duplicate tubes after subtracting the blank value. The cells (7.0 x 10<sup>6</sup> cells/ml HBSS) were incubated with the [<sup>14</sup>C]UCA isomers at 4 °C for 30 min, washed, and transferred into scintillation vials. Control vials without cells, designated as “total UCA”, underwent a similar incubation to eliminate any non-specific binding effect by the incubation tubes. *Hatched symbols*, Estimated cpm values have been used for total UCA samples with >10<sup>7</sup> cpm due to technical maximum count limit.

Figure 3 shows displacement of [<sup>14</sup>C]*cis*-UCA incorporation by non-labeled *cis*-UCA. Neutrophils isolated from freshly drawn venous blood of a single volunteer were assayed at two occasions (seven days apart) for displacement at pH 7.4. The cells (7.4 x 10<sup>6</sup>/ml in Exp 1 and 6.3 x 10<sup>6</sup>/ml in Exp 2) were incubated in HBSS containing 1 mM [<sup>14</sup>C]*cis*-UCA with or without 10 mM non-labeled *cis*-UCA (total

volume 200 µl) at the indicated temperatures for 1 h. In Exp 1, the cells were washed after incubation as a pellet only, whereas the cells were resuspended in washing medium (HBSS) in Exp 2. The data are from triplicate incubations.

5 Figure 4 shows the distribution of incorporated *cis*-UCA in cellular fractions and by incubation temperature. *A*, Proportional [<sup>14</sup>C] activity in different fractions of cells incubated at 4 °C (mean ± SEM, n=4 independent experiments). Neutrophils (50–200 x 10<sup>6</sup> cells/ml) isolated from buffy coats were incubated with 1 or 5 mM [<sup>14</sup>C]*cis*-UCA at 4 °C for 20–30 min. The cells were disrupted by sonication, the 10 cellular fractions were separated by sucrose ultracentrifugation, and the bound activity was measured in each fraction. *B*, Effect of incubation temperature on the uptake of *cis*-UCA to neutrophils of the peripheral blood (mean ± SEM, n=4 independent experiments). *C*, Distribution of *cis*-UCA in cellular fractions according to incubation temperature (mean ± SEM of duplicate incubations).

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Figure 5 shows the elution of cytosol-associated [<sup>14</sup>C]UCA in S-200 gel filtration. Neutrophils (160–190 x 10<sup>6</sup>) were incubated with 1 mM [<sup>14</sup>C]UCA isomers at 4 °C for 20 min, washed, lysed, and fractionated with sucrose ultracentrifugation. The cytosolic fraction was applied to the gel, and the protein content and UCA activity 20 were measured in the elute. *A*, Cytosol of cells incubated with [<sup>14</sup>C]*cis*-UCA. *B*, Cytosol of cells incubated with [<sup>14</sup>C]*trans*-UCA. *C*, Elution of standard molecular weight protein markers and *cis*-UCA alone in the same conditions.

25 Figure 6 shows lack of UCA metabolism in the experimental conditions. Cytosolic proteins of [<sup>14</sup>C]UCA-labeled neutrophils from various binding assays were precipitated with 10 % TCA on ice overnight. The amount of radioactive label was measured in the precipitate and protein-free supernatant by scintillation counting and the content of intact (non-metabolized) UCA isomers in the supernatant by HPLC. The data represent results from a set of whole-cell incubations with *cis*-UCA 30 (n=7) and *trans*-UCA (n=2) and from two experiments with post-lysis incubation with both isomers (arrow). Pearson's correlation coefficients and p-values have been calculated for both isomers.

Figure 7 shows the pH effect of UCA isomers on standard incubation buffers. The pH was measured in PBS, pH 7.0 (*A*), and in HBSS buffers, pH 7.4 (*B*), containing graded concentrations of *cis*-UCA and *trans*-UCA.

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Figure 8 shows the relation of respiratory burst activity and acidification of the cytosol by UCA isomers. In three independent experiments, neutrophils were incubated with 3 mM *cis*- or *trans*-UCA and analyzed simultaneously for respiratory burst chemiluminescence and pH indicator fluorescence. *A*. Intracellular pH indicator dye fluorescence compared to control levels at the same extracellular pH. The cells were loaded with BCECF, washed, incubated with UCA, and analyzed with flow cytometry. The percentages have been calculated from the geometrical mean fluorescence intensities. *B*. Respiratory burst responses compared to control levels without UCA at the same pH. The results are from two parallel assays within each of the three experiments. In *A* and *B*, the pH of the extracellular medium was adjusted to 6.5 or 7.4 after adding UCA. *C*. Respiratory burst response with UCA as a function of extracellular pH. The data are from the three experiments above complemented with simultaneous incubations where pH was measured only but not adjusted after the addition of 3 mM UCA. *D*. Dependence of respiratory burst on intracellular acidification. Correlation coefficients for *cis*- ( $p=0.048$ ,  $n=12$ ) and *trans*-UCA ( $p=0.065$ ,  $n=11$ ) were calculated from the same experiments as in *B*.

Figure 9 shows the intracellular pH calibration in UCA-treated neutrophils *in situ*. BCECF-labelled cells were used as pH reference cells after treatment with proton 25 ionophore nigericin in high-potassium Pipes buffer at various pH. Other BCECF-labelled cells were incubated with or without 3 mM UCA in low-potassium Pipes buffer adjusted to the same pH levels as those of the calibration buffers. Intracellular pH was calculated using BCECF median fluorescence intensity obtained in flow cytometry.

## DETAILED DESCRIPTION OF THE INVENTION

According to a preferable embodiment, the pharmaceutically acceptable agent is an acid having its dissociation constant in the range 6.7 to 7.4, preferably in the range 5 6.9 to 7.3; most preferably about 7.0.

The acid is preferably cis-uropionic acid or salt thereof, but it is not restricted hereto. Any other pharmaceutically acceptable, non-toxic agent having its dissociation constant in the range defined above and being able to accumulate inside a cell 10 would be useful. Such agents may be inorganic or organic, preferably an organic acid having, like cis-uropionic acid, a heterocyclic ring to which a saturated, or more preferably, an unsaturated carboxylic acid moiety is attached. The heterocyclic group may be, for example, an imidazole (as for cis-uropionic acid) or any other heterocyclic or poly-heterocyclic group having the ability to donate a proton at 15 cytoplasmic pH and thereby acidify the cytoplasm. As examples of other suitable heterocyclic groups can be mentioned thiazole, thiophene, furan, oxazole, triazole, tetrazole, pyrazole, pyridine, pyrimidine and triazine.

The pharmaceutically acceptable agent is admixed with a carrier, which can be one 20 single component, or more preferably, a mixture of two or more components. One of the components is suitably a buffering agent, which adjusts the pH of the composition to the desired value.

Especially when cis-uropionic acid is the active agent, it is preferable to adjust the 25 pH of the composition to 6.5 to 7.0, preferably 6.7 to 7.0. In this pH range, cis-uropionic acid is still non-dissociated, while trans-uropionic acid is fully dissociated. Such a composition will therefore be specific with respect to cis-uropionic acid.

As examples of suitable buffering agents to adjust pH to 6.5-7.0 can be mentioned 30 50 mM sodium phosphate supplemented with 55 mM sodium chloride, 50 mM sodium citrate supplemented with 120 mM sodium chloride, and 10 mM Pipes supplemented with 133 mM sodium chloride.

The method and composition according to this invention is useful for treatment or prevention of any disease or disorder curable by increased immunosuppression. The term immunosuppression used herein refers to regulation, typically down-regulation, of the body's immune system by affecting to the activity and function of the cells of the immune system in a way which prevents the undesired adverse effects of an immune response. Examples of the target cells of the method and compositions of the present invention are granulocytes (neutrophils, eosinophils, basophils), NK-cells, T- and B-lymphocytes, monocytes, macrophages, mast-cells and antigen presenting cells, such as dendritic cells, and their precursor cells and specific functional and phenotypic subsets. Most preferably the target cells of the method and compositions of the present invention are cells of the innate immune system, such as neutrophils and NK-cells.

It is well established that the appropriate function of cells of the immune system is vital for host's survival against invading pathogens, parasites and even physical hazards (e.g. microscopic particles inhaled) found in the living environment. Normally, immune cells recognize, isolate and eliminate locally infectious/damaging agents in a well-orchestrated process. For this purpose, the immune cells are armed with various biochemical response mechanisms, which become active during the infectious attack. For example, neutrophilic leukocytes, neutrophils, contain a highly specific enzyme complex, NADPH oxidase system, which, when triggered upon cell activation, is able to generate large amount of toxic oxygen metabolites, which can exert a number of damaging effects against biological material, and may also act as proinflammatory signals for other cells types. In general, leukocyte activation leads to a local inflammatory reaction which is an essential part of host's immune response and which promotes the resolution of the infectious assault and initiates the healing process. However, if normal host tissues are inappropriately identified as foreign or damaged structures, or due to the hyperactivation of host's immune system associated with some pathological states, normal tissue is attacked by immune cells which elicit their full destructive potential against host itself. As examples of such states can be mentioned groups of conditions such as local and systemic inflammatory diseases, autoimmune diseases

- and allergic conditions. As examples of specific diseases or disorders can be mentioned hypersensitivity reactions such as contact hypersensitivity or delayed type hypersensitivity. Preferably the condition which can be treated or prevented by the method and compositions of this invention is a local or systemic inflammatory reaction which involves the activation of the preferable target cells, such as inflammatory conditions of the skin, including psoriasis, acute or chronic dermatitis; inflammatory conditions of mucous membranes or the connective tissue of the oral cavity, eyes and genitals, such as periodontitis, conjunctivitis, vaginitis; inflammatory conditions of mammary glands, including mastitis; or any other local or systemic condition manifesting a recognized inflammatory component in the disease pathogenesis or progression, such as vasculitis, acute graft rejection, chronic obstructive pulmonary disease, asthma, reperfusion injury, and sepsis associated tissue damage. However, the conditions that can be treated or prevented according to this invention are not restricted to the aforementioned examples.
- For the purpose of this invention, the pharmaceutically acceptable agent can be administered by various routes, either systemically or locally. The suitable administration forms include, for example, oral formulations; parenteral injections including intravenous, intramuscular, intradermal and subcutaneous injections; and mucosal, topical, transdermal, inhalation, nasal or rectal formulations. Particularly suitable formulations are formulations for local delivery such as topical formulations in the form of ointments, gels, creams, pastes, solutions, suspensions, lotions and emulsions.
- The required dosage of the pharmaceutically acceptable agent will vary with the particular condition being treated, the severity of the condition, the duration of the treatment, the administration route and the specific compound being employed. In a topical formulation the amount of the pharmaceutically acceptable acid can typically range from 0.01 % to 50 %, preferably in the range 0.1 to 10 %.
- The invention will be illuminated in detail in the following Experimental Section.

## EXPERIMENTAL SECTION

The aim of the present study was to investigate the binding of radiolabeled UCA in a model cell, the human peripheral blood neutrophil, which has been shown to be affected by UCA (Kivistö 1996). Instead of being able to demonstrate a typical ligand-receptor interaction, we found that UCA has an exceptional binding property, which leads to rapid and irreversible accumulation of intact UCA into the cytosol. Urocanic acid is an UV radiation-absorbing substance in the mammalian skin. The *cis*-UCA is an immunosuppressant in animal models *in vivo*, but the target cell type(s) and mode(s) of action have remained obscure. We investigated the binding and the site of action of UCA in live human polymorphonuclear neutrophils, an immune cell type whose function is known to be affected by UCA and which is known to play a major role in inflammatory reactions. We observed a linearly concentration-dependent accumulation of radiolabeled *cis*- and *trans*-UCA up to unexpectedly high incubation concentrations ( $\geq 30$  mM) with almost 95 % of the cell-bound fraction concentrating in the cytosol. Because the isomers appeared in an unbound and non-metabolized form in the cytosol, we questioned whether UCA could act through a mechanism different from conventional receptor/protein-ligand interaction. The isomers affected intracellular pH. FACS analyses showed that acidification of the intracellular compartments of neutrophils by *cis*-UCA at extracellular pH 6.5 was significantly greater than by *trans*-UCA ( $p=0.00031$ ), whereas the isomers did not acidify at pH above neutral. In the same conditions at pH 6.5, *cis*-UCA inhibited the respiratory burst activity of neutrophils more than *trans*-UCA ( $p=0.023$ ). Stereospecificity of this type could be explained by dissimilar  $pK_a$  values of the two isomers, and we propose a model for *cis*-UCA action through intracellular acidification. We conclude that *cis*-UCA may suppress innate immunity by inhibiting neutrophil activation and function through intracellular acidification in an extracellular pH 6.1–7.0 window.

## Methods

### Urocanic acid and synthesis of [<sup>14</sup>C]-labeled isomers

- 5     *Trans*-urocanic acid (*trans*-UCA, 3-(1*H*-imidazol-4-yl)-2-propenoic acid) was purchased from Sigma (St. Louis, MO, USA). *Cis*-UCA was prepared from *trans*-UCA with UV photoisomerization (see below). The chemical purity of the UCA isomers was above 99.7 % by high-pressure liquid chromatography (HPLC).
- 10   The synthesis of [<sup>14</sup>C]-radiolabeled *trans*- and *cis*-UCA is outlined in Fig. 1. We started the synthesis of [<sup>14</sup>C]*trans*-UCA (6) by condensing formamidine acetate (1) and dihydroxyacetone (2) in liquid ammonia to give (3), utilizing the procedure of Griffith *et al.* (1983) with several modifications. After neutralization of (3) to the free base (4), it was oxidized to 4-imidazolecarbaldehyde (5) (Lindgren *et al.*, 1980). Condensation of (5) with [2-<sup>14</sup>C]malonic acid (Amersham Pharmacia, Little Chalfont, UK) under Knoevenagel conditions using a modified method of Morrison *et al.* (Mohammad 1991) afforded *trans*-UCA (6). Compound (6) (138 mg, 1 mmol) was dissolved in water (500 ml). The solution was brought to pH 9 with solid potassium hydroxide and then irradiated under nitrogen atmosphere at 10 °C for 4 h.
- 15   Condensation of (5) with [2-<sup>14</sup>C]malonic acid (Amersham Pharmacia, Little Chalfont, UK) under Knoevenagel conditions using a modified method of Morrison *et al.* (Mohammad 1991) afforded *trans*-UCA (6). Compound (6) (138 mg, 1 mmol) was dissolved in water (500 ml). The solution was brought to pH 9 with solid potassium hydroxide and then irradiated under nitrogen atmosphere at 10 °C for 4 h.
- 20   Photoisomerization was performed in a Normag falling-film photoreactor with Hanau quartz mercury high-pressure lamp (500 W, 270–350 nm, water as solvent). The resulting mixture (*trans/cis* ca. 30/70 by HPLC) was evaporated to dryness and the residue dissolved in 12.5 mM acetic acid. This solution was adjusted to pH 9 and chromatographed on an ion exchange column (25 x 2.3 cm, 200–400 mesh,
- 25   acetate form, Bio-Rad 1-x8) using 12.5 mM (500 ml), 25 mM (500 ml), and 100 mM (1000 ml) acetic acid as successive eluents. *Cis*-UCA appeared after ca. 1100 ml and *trans*-UCA mainly after 1300 ml eluent volumes. Removal of the solvent from the fractions, followed by washing with diethyl ether and drying *in vacuo* at 65 °C over phosphorus pentoxide, yielded the pure [<sup>14</sup>C]*trans*- and [<sup>14</sup>C]*cis*-isomers (6) and (7). The yield of (6) from the preceding step was 35 mg (25 %), mp. 226 °C. The chemical purity of the product (6) by HPLC (see below) was above 99.8 %, and the specific activity was 2.2 mCi/mmol. The corresponding yield of (7) was 85 mg

(58 %), mp. 176–178 °C. HPLC analysis indicated the material to be more than 99.5 % chemically pure with a specific activity of 5.8 mCi/mmol. When used in the experiments, the radiolabeled and non-labeled *cis*- and *trans*-isomers were dissolved directly in the incubation buffers up to 100 mM and 30 mM concentrations,  
5 respectively. The dissolution of *trans*-UCA was aided with gentle warming in a water bath when needed.

#### HPLC analysis of UCA

10 An aminopropyl stationary phase column Lichrosorb NH<sub>2</sub>, Hibar RT, 250 x 4 mm, 5 µm (Merck, Darmstadt, Germany) was used. The eluent was a 50 % (v/v) mixture of acetonitrile and a solution of 2 % (v/v) acetic acid and 0.5 % (w/v) ammonium acetate in water (pH *ca.* 5). The isomers were detected at 268 nm, and the retention times were T<sub>r</sub>(*cis*) 3.7 min and T<sub>r</sub>(*trans*) 5.4 min.  
15

#### Scintillation counting

Samples were mixed with OptiPhase HiSafe 2 scintillation liquid (EG&G Wallac, Turku, Finland) and [<sup>14</sup>C]UCA radioactivity measured in RackBeta 1214

20 scintillation counter (EG&G Wallac). The counting efficiency was 96.7 % ± 0.12 % (mean±SEM, n=48).

#### Purification of neutrophils

25 Peripheral blood neutrophils were isolated from heparinized blood or buffy coats of healthy donors. Erythrocytes were sedimented with 6 % dextran T-500 (Pharmacia, Sweden). Neutrophils were separated from the leukocyte-rich dextran plasma by centrifugation on Ficoll-Hypaque (Pharmacia), purified by hypotonic lysis of remaining erythrocytes, and washed with Ca- and Mg-free HBSS. For the  
30 intracellular pH experiments, neutrophils were prepared without erythrocyte lysis. The cells, media and centrifuges were kept at room temperature during cell preparation to avoid temperature fluctuations. By flow cytometry analysis, 99.6 %

of the separated neutrophils were CD11b<sup>+</sup>/CD35<sup>+</sup>, 98% CD45<sup>+</sup>, 98 % CD62L<sup>+</sup>/CD32<sup>+</sup>, 2.0 % HLA-DR<sup>+</sup>, 2.1 % CD3<sup>+</sup>, 1.0 % CD8<sup>+</sup>, 1.2 % CD4<sup>+</sup>, and 0.8 % CD14<sup>+</sup> cells.

5 Assay for respiratory burst activity

The respiratory burst activity was used as a measure of neutrophil function. The UCA isomers were tested in a chemiluminescence assay with opsonized zymosan as described (Kivistö et al. 1996). The peak values were recorded.

10

Whole-cell binding assays

- Isolated neutrophils were resuspended in HBSS, pH 7.4, at 2–10 x 10<sup>6</sup> cells/ml. The [<sup>14</sup>C]*cis*- or [<sup>14</sup>C]*trans*-UCA stock solutions were added to yield a concentration range 0.1 μM–30 mM, and the tubes, in duplicate, were incubated at 4 °C (or at 25 °C and 37 °C) for 30 min. The cells were then washed once with ice-cold HBSS and transferred into liquid scintillation vials. The total [<sup>14</sup>C]UCA activity in the incubation tubes was determined by measuring samples from each standard concentration, and blank scintillation values were subtracted before data analysis.
- 15 Some binding experiments were performed in 50 mM sodium citrate/120 mM NaCl buffer, pH 6.5, as indicated, using the same buffer in washing steps.
- 20

Preparation of neutrophil cytosol and membrane fractions

- 25 The localization of the cell-bound UCA in membrane, cytosol and nucleus was investigated after the incubation of whole cells with [<sup>14</sup>C]*cis*-UCA as described above. After washing with HBSS, the cells were suspended (200 x 10<sup>6</sup> cells/ml) in ice-cold lysis buffer containing 10 mM Pipes, 10 mM KCl, 3 mM NaCl, 4 mM MgCl<sub>2</sub>, pH 7.0, supplemented with 0.5 mM PMSF, 10 μM leupeptin, and 10 μM pepstatin A (all from Sigma) as proteinase inhibitors. The cell membranes were broken by sonication on ice. The lysate was centrifuged (800 x g, 25 °C, 10 min), and the post-nuclear supernatant was layered on discontinuous cushions of sucrose
- 30

in lysis buffer. After ultracentrifugation ( $120\,000 \times g$ , 4 °C, 45 min), the cytosol, membrane, and nuclei/debris fractions were recovered by careful pipetting, and [ $^{14}\text{C}$ ] activity was measured.

5     Gel filtration of neutrophil cytosol

For the macrofractionation of cytosol proteins, the sample (0.5–2.5 ml) was applied to the balanced Sephadryl S-200 gel filtration (Pharmacia) column at 4 °C, and the proteins were eluted with PBS, pH 7.0, at a flow rate of about 0.6 ml/min. The  
10    elution of proteins was followed with a flow-through UV monitor at 254 nm and a potentiometric recorder. A typical run consisted of thirty 6-ml fractions and lasted for almost six hours. The elution volumes of proteins of different molecular weights were determined with a cocktail of standard proteins and peptides of 0.6–2000 kDa size.

15

Protein concentration assay

Protein concentration was determined with Bio-Rad (Munich, Germany) protein assay using bovine albumin as a standard.

20

Monitoring of intracellular pH

Intracellular pH levels in neutrophils were monitored with flow cytometry utilizing a pH-sensitive fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-  
25    carboxyfluorescein (BCECF, acetoxyethyl ester; Molecular Probes, Leiden, The Netherlands). About  $30 \times 10^6$  cells were incubated in 10 ml HBSS, pH 7.4, containing 0.35 μM BCECF at 25 °C for 30 min, washed twice in HBSS, and resuspended in 1 ml of 154 mM NaCl. Aliquot (235 μl) of the incubation medium with known UCA concentrations and checked pH was applied to the cells ( $4.5 \times 10^5$   
30    cells/15 μl NaCl) in polystyrene tubes, incubated at 25 °C for about 20 min, and analyzed in a flow cytometer.

Calibration of intracellular pH was performed *in situ* using the K<sup>+</sup>/H<sup>+</sup> ionophore nigericin. An excess of pH calibration buffers (10 mM Pipes, 131 mM KCl, pH adjusted to 6.10, 6.50, 6.80, 7.10, 7.40, and 7.60 or 7.70) and 10 µM nigericin was added to the BCECF-labelled cells in 154 mM NaCl. The cells were kept at room 5 temperature and analysed by flow cytometry within 45 min. Cells incubated with or without UCA were analysed for intracellular pH simultaneously. The pH was adjusted to the same values as those in the calibration buffers. Intracellular pH was determined from a BCECF fluorescence intensity calibration curve.

#### 10 Statistical analysis

The results have been presented as mean ± SEM. Statistical significance of data in the binding studies and functional tests were calculated with two-way Student's *t* test. The Pearson's correlation coefficients were determined for UCA isomer 15 concentrations detected by HPLC and scintillation counting of cell samples. The *p*-values for correlation were determined after Fisher's Z transformation.

### Results

#### 20 UCA accumulates in neutrophil cytosol

Radioactive, [<sup>14</sup>C]-labeled UCA isomers were synthesized to examine the binding of UCA to isolated human peripheral blood neutrophils. The cells, incubated with UCA in HBSS at 4 °C for 30 min, incorporated both isomers in a linear dose-dependent manner over the studied concentration range of 100 nM to 30 mM (Fig. 2). The proportion of total binding was 4.5 % ± 1.1 % (range 2.9–6.6 %) for *cis*-UCA and 7.1 % ± 3.2 % (range 3.7–17 %) for *trans*-UCA (n=12 measurements in duplicate for both isomers). An interesting feature of this uptake was that we were unable to demonstrate displacement of the [<sup>14</sup>C]UCA radiolabel with non-labeled 30 (“cold”) UCA as one would expect in conventional ligand-receptor binding (Fig. 3).

To investigate the distribution of the cell-bound UCA in the cytosol, cell membrane, and nuclear compartments, the cells were first incubated with radiolabeled UCA as above, and then they were lysed and fractionated on 120.000 x g sucrose cushions. The contents in the ultracentrifuge tubes were divided into cytosolic, membrane and 5 nuclear fractions. The volume of each fraction was determined accurately. Then the [<sup>14</sup>C]UCA activity in aliquots of the fractions was measured, and the total UCA content was calculated for each fraction. Independent incubation experiments (*n*=4) showed that 92.0 % ± 2.2 % of the neutrophil-incorporated *cis*-UCA was recovered in the cytosol (Fig. 4A). Binding to membranes (mean 2.7 % ± 1.8 %) was 10 significantly lower than what was found in the cytosol (*p*=3.7 x 10<sup>-5</sup>). The remaining cell-bound *cis*-UCA (5.3 % ± 2.1 %) was detected in the nuclear (and possibly non-lysed cell) fraction of the cell lysate (Fig. 4A).

#### UCA is not bound to cytosolic proteins

15

As most of the UCA that incorporated in the cells appeared in the cytosol, we determined if the cytosolic UCA was bound to molecular components of the neutrophil cytosol. Cytosol of [<sup>14</sup>C]UCA-preincubated cells was separated with sucrose ultracentrifugation and applied into S-200 gel filtration column. Cytosol 20 fractions were then collected and the radioactivity was measured in each fraction. As shown in Fig. 5A and 5B, [<sup>14</sup>C] activity was found in low-molecular-weight fractions containing no detectable protein. This elution pattern was identical to a run where [<sup>14</sup>C]*cis*-UCA alone was applied into the gel filtration column (Fig. 5C), suggesting that UCA is not bound to any major soluble protein fraction in 25 neutrophil cytosol.

An additional, post-lysis labeling test was carried out to verify the results from the experiments with [<sup>14</sup>C]UCA-preincubated cells. In this test, non-labeled neutrophil cytosol was separated as described and aliquots of the cytosol were then incubated 30 with 5 mM *cis*- or *trans*-[<sup>14</sup>C]UCA overnight on ice. The cytosol was then fractionated on S-200. No protein-associated [<sup>14</sup>C]UCA activity was observed, and elution profiles similar to pre-lysis incubation were recorded. Thus, the main

soluble protein fractions in the neutrophil cytosol were shown to be incapable of binding accumulated UCA before and after cell lysis.

UCA remains intact in neutrophil cytosol

5

Next, we examined whether the [<sup>14</sup>C]UCA in the cytosol was metabolized by neutrophils after the uptake by determining how much of the radioactive label was associated with intact UCA. This was carried out by precipitating the cytosolic proteins of [<sup>14</sup>C]UCA-labeled neutrophils with 10 % trichloroacetic acid (TCA) on 10 ice overnight. The amount of radioactive label was then measured both in the precipitate and in the protein-free supernatant by scintillation counting, and the content of intact UCA in the supernatant by HPLC. All [<sup>14</sup>C]UCA activity was found in the supernatant, the recovery being 102 % ± 3.9 % (*n*=9) for *cis*-UCA and 100.2 % ± 0.9 % (*n*=4) for *trans*-UCA when the radioactivity in the cytosol 15 immediately after the addition of TCA and after spinning down the protein precipitate was compared. No radioactivity was found in the protein pellet. More importantly, the chromatographically determined concentrations of intact *cis*- and *trans*-UCA correlated with concentrations achieved by scintillation counting in the same samples (Fig. 6), indicating that UCA isomers were not metabolized in 20 neutrophil cytosol. No endogenous UCA could be found by HPLC analysis in cells that were not pretreated with UCA isomers (data not shown).

UCA lowers extracellular and intracellular pH

25 The results reported so far show that instead of behaving like a typical cell-surface receptor agonist, UCA accumulates in high concentrations inside a neutrophil, where it is not bound to soluble intracellular proteins nor subject to significant metabolism. As such, UCA resembles small ions (e.g., K<sup>+</sup>, Na<sup>+</sup>, H<sup>+</sup>, Cl<sup>-</sup>) which enter the cell and modulate cell functions by altering the physico-chemical micro- 30 environment (pH, ion potential, ion strength) of the cytosol. Therefore, we hypothesized that the high levels of intact UCA may provoke cellular changes simply due to its passive presence in the cytosol as an acid. The pK<sub>a</sub>'s being around

- 4.0 and 6.1 for *trans*-UCA (Roberts *et al.* 1982, Krien & Kermici 2000) and 3.3 and 7.0 for *cis*-UCA (Roberts *et al.* 1982), one possible mode of action could be the acidification of the cytosol at physiological pH. Such a possibility was approached by testing the effect of UCA on pH first in a buffer solution and then in intact cells.
- 5 The isomers lowered the pH in a standard PBS buffer, pH 7.0, in a dose-dependent manner at concentrations above 1 mM (Fig. 7A). When UCA isomers were added in HBSS buffer, pH 7.4, concentrations above 1 mM again dropped the pH dose-dependently (Fig. 7B). Interestingly, when the pH of HBSS buffer solution was adjusted to 6.5 prior to UCA addition, *i.e.* below the second pK<sub>a</sub> of *cis*-UCA, only
- 10 *trans*-UCA was able to markedly reduce the pH (Fig. 7B), suggesting that *cis*-UCA is only partly deprotonated at this pH.

To test the effect of UCA on intracellular pH, neutrophils were loaded with the fluorescent pH-indicator dye BCECF, and the fluorescence of UCA-treated cells 15 was measured with FACS. As the data above indicate, UCA itself can lower the pH of the test solution depending on isomer and initial pH of the solution. On the other hand, it is well known that the intracellular pH is affected by the pH of the environment. Therefore, in order to avoid the artefact that the acidification of the test solution by UCA addition might affect intracellular BCECF fluorescence, we 20 adjusted the pH of the test solution back to the original pH after the addition of UCA. In these pH-controlled conditions, 3 mM *trans*- and *cis*-UCA had no significant effect on the intracellular BCECF signal at pH 7.4 (Fig. 8A, lower bars). In contrast, when the pH was adjusted to 6.5, *cis*-UCA caused a significant reduction by  $15\% \pm 4.0\%$  ( $p=0.022$ ,  $n=4$ , paired *t* test) in the fluorescence signal as 25 an indication of cytosolic acidification (Fig. 8A, upper bars). Figure 8 shows data from three independent experiments measuring simultaneously BCECF signals and respiratory burst activity of the cells (see below). A fourth experiment was performed for intracellular BCECF fluorescence measurement only. Also *trans*-UCA decreased the fluorescence signal significantly by  $9.4\% \pm 4.1\%$  at pH 6.5 30 ( $p=0.032$ ,  $n=4$ ), but the effect was less pronounced. However, the difference in proportional BCECF fluorescence reduction between 3 mM *cis*-UCA and *trans*-UCA was highly significant ( $p=0.00031$ ,  $n=4$ ) (Fig. 8A, upper bars).

To achieve a more specific view of the ability of *cis*-UCA to acidify the cytosol, the exact intracellular pH was determined by the use of the K<sup>+</sup>/H<sup>+</sup> ionophore nigericin. Incubation of neutrophils with UCA in buffered solutions of several pHs in the range 6.1–7.7 demonstrated that 3 mM *cis*-UCA lowers the intracellular pH in dose- and extracellular pH-dependent manner below pH 7, whereas *trans*-UCA has only a minor effect (Fig. 9). The 0.3 mM concentration of *cis*-UCA had a much smaller effect in the same pH range (not shown).

UCA inhibits neutrophil respiratory burst stereospecifically and pH-dependently

The data shown above suggest that in a slightly acidic environment only *cis*-UCA is able to markedly decrease cytosolic pH whereas at physiological pH neither *trans*- nor *cis*-isomer had any effect. To examine how the cytosol-acidifying effect of UCA correlates with the previously reported inhibition of neutrophil respiratory burst activity, we measured the effect of 3 mM UCA on opsonized zymosan-induced chemiluminescence by the same batch of neutrophils and in the same experimental conditions described above, i.e. when the pH of the test solution was adjusted back to its initial level after UCA addition. As shown in Fig. 8B, *trans*-UCA had no effect on chemiluminescence at pH 7.4, whereas an inhibition of 14 % ± 4.0 % (n=3) was observed at pH 6.5. In the same conditions, *cis*-UCA suppressed the respiratory burst activity by 15 % ± 8.4 % and 44 % ± 1.3 %, respectively. Interestingly, when the pH of the test solution was left unadjusted after UCA supplementation, *trans*-UCA inhibited the chemiluminescence by 31 % ± 8.4 % and 48 % ± 4.0 % at pH 6.22 ± 0.02 (nominal pH 7.4) and 5.87 ± 0.06 (nominal pH 6.5), respectively. The corresponding inhibitions for *cis*-UCA were 41 % ± 10 % at pH 6.60 ± 0.02 (nominal pH 7.4) and 48 % ± 1.6 % at pH 6.33 ± 0.07 (nominal pH 6.5).

When the obtained respiratory burst response data is plotted against the measured pH in the incubation medium, it is evident that lowering the extracellular pH suppresses respiratory burst activity in the presence of 3 mM UCA (Fig. 8C). The plot also demonstrates that *cis*-UCA possesses a more prominent inhibitory activity on the cells in the extracellular pH range 6.1–7.0 than *trans*-UCA, whereas no

difference can be found at above pH 7. When the respiratory burst activity is calculated as a function of the respective intracellular BCECF fluorescence in the same cells, it can be observed that the suppression of respiratory burst activity is related to the decrease in intracellular pH produced by UCA isomers through either 5 extracellular or intracellular acidification (Fig. 8D).

### **Conclusions**

Because UCA is a weak organic acid, the accumulation of UCA inside the cell 10 could regulate the cytosolic pH. This, however, greatly depends on the protonation status of the entering UCA molecules. UCA is a polyprotic acid with two proton-donor moieties, the carboxyl group and the imidazolyl group. The pK<sub>a</sub> of the carboxyl group is 4.0 for *trans*-UCA and 3.3 for *cis*-UCA (Roberts 1982), from which it follows that practically all UCA molecules are deprotonated at the carboxyl 15 group at pH above 4, according to the Henderson-Hasselbalch Equation (H-H Eq.). Therefore, at the physiological pH range, the protonation status of the imidazolyl group alone determines whether the molecule is able to donate a proton and thereby promote acidification. The imidazolyl pK<sub>a</sub> of *trans*-UCA is 6.1 (Roberts *et al.* 1982, Krien & Kermici 2000) while for *cis*-UCA it is markedly higher, 7.0, potentially 20 due to the stabilized tautomeric form of the *cis*-isomer caused by intramolecular hydrogen bonding between the carboxyl and imidazolyl moieties (Roberts 1982). Consequently, only at pH 7.0 and above, the imidazolyl group of *cis*-UCA favors 25 deprotonation, whereas *trans*-UCA is almost completely deprotonated at the same pH. In the present study, this was clearly demonstrable by an experiment where the addition of *trans*-UCA in HBSS buffer adjusted to pH 6.5 dropped the pH while *cis*-UCA had almost no effect.

It can be hypothesized that the ability of UCA to acidify cytosol in living cells 30 depends on two major parameters: the pH of the extracellular space and the initial pH of the cytosol. Because UCA is found mainly in the skin, one should consider these two parameters in the context of the physiological environment. It is well known that the human skin has an acid mantle with a superficial pH around 4–6.

When the stratum corneum is stripped layer by layer, the pH increases gradually and, after total removal of the stratum corneum, the pH in the remaining epidermis is about 6.9 (Öhman & Vahlquist, 1994). In deeper layers, the almost neutral pH of the interior body is reached. A recent analysis provides evidence that UCA is the  
5 major pH-regulating factor in the human stratum corneum (Krien & Kermici 2000). The majority of UCA resides in the stratum corneum; however, a significant amount of UCA diffuses into and evidently also through the (epi)dermis, because elevated levels of *cis*-UCA can be detected in the urine within 1–4 h following total-body UVB exposure (Kammayer *et al.* 1997). Concerning the intracellular environment,  
10 pH in the resting neutrophil cytosol is 7.0–7.4, *i.e.* above the imidazolyl pK<sub>a</sub>, which suggests that both UCA isomers exist mainly in the deprotonated state in the neutrophil cytosol. At an extracellular pH above the imidazolyl pK<sub>a</sub> (6.1 for *trans*-UCA and 7.0 for *cis*-UCA), the majority of UCA molecules would be in the deprotonated form and no significant acidification would occur after entering the  
15 cytosol. In contrast, at a pH below the imidazolyl pK<sub>a</sub>'s, UCA would be mainly in the protonated form capable of promoting cytosolic acidification upon cell entry. Moreover, according to the H-H Eq., it can be speculated that the amount of UCA-associated protons and thus the reduction of cytosolic pH would be directly proportional to the transmembrane pH difference between the cytosol and the acidic  
20 extracellular environment. To provide experimental support for these hypotheses, we measured the change in cytosolic pH in UCA-treated cells. When extracellular pH was strictly controlled to 7.4 in the incubation mixture *i.e.* above the pK<sub>a</sub>'s of imidazolyl group of both UCA isomers, no acidification was seen, as one could expect. On the other hand, at controlled pH 6.5, *cis*-UCA with imidazolyl pK<sub>a</sub> of 7.0  
25 clearly decreased the cytosolic pH while *trans*-UCA (pK<sub>a</sub> 6.1) had only a minor effect. This was also predictable from a calculation using the H-H Equation: at pH 6.5 over 70 % of *cis*-UCA is in protonated and 70 % of *trans*-UCA in the deprotonated state. In theory, lowering the extracellular pH below 6.1 would have allowed us to detect a *trans*-UCA-induced fall in the cytosolic pH, but it was not  
30 possible to test this with the BCECF dye due to its limited operational pH range. Taken together, it is evident that at slightly acidic environment, such as in the upper viable layers of the epidermis, *cis*-UCA, in effect, can act as a proton shuttle to

reduce cytosolic pH. This unique property of UCA originates from a shift in the imidazolyl pK<sub>a</sub> caused by changed spatial structure of UCA upon *trans*-to-*cis* photoisomerization.

- 5 There is no previous data in the scientific and patent literature suggesting that UCA preparations should be formulated at the pH range proposed in the present invention. In the US patent 5,494,676 by Stab et al. it was described that the photoisomerisation reaction of 1 % trans-UCA was performed in a water solution, where the pH was adjusted to 6.9 with NaOH prior the irradiation with an UV-lamp.
- 10 This solution, containing equal amounts trans-UCA and cis-UCA, was then used to prepare topical O/W-cream formulations. However, the pH of the topical preparations was not pH-adjusted, nor pH-buffered to the preferred pH-range of the present invention.
- 15 In conclusion, the present study shows data which, for the first time, may explain the stereospecific action of UCA on immune cells *in vivo*. Paradoxically, modulation of cell function by UCA seems not to depend directly on stereoisomerism but rather on a subtle but critical change in the acid-base properties of the molecule after photoconversion from the *trans*- to *cis*-UCA.

20

The invention is further illuminated by the following non-restricting Examples.

#### EXAMPLES OF FORMULATIONS ACCORDING TO THE INVENTION

25 **Gel Composition 1 (% w/w)**

|                    |        |
|--------------------|--------|
| Cis-urocanic acid  | 0.1-10 |
| Carbopol 974       | 1.5    |
| Propylene glycol   | 12.5   |
| 30 Buffering agent | 0.01-1 |
| Purified water to  | 100    |

**Gel Composition 2 (% w/w)**

|                                  |        |
|----------------------------------|--------|
| Cis-urocanic acid                | 0.1-10 |
| Natrosol (hydroxyethylcellulose) | 1.0    |
| 5 Buffering agent                | 0.01-1 |
| Purified water to                | 100    |

**Cream Composition 1 (% w/w)**

|   |                       |        |
|---|-----------------------|--------|
|   | Cis-urocanic acid     | 0.1-10 |
|   | Propylene glycol      | 50     |
| 5 | Cetostearyl alcohol   | 15     |
|   | Sodium lauryl sulfate | 1      |
|   | Buffering agent       | 0.01-1 |
|   | Purified water to     | 100    |

**10 Cream Composition 2 (% w/w)**

|    |                        |        |
|----|------------------------|--------|
|    | Cis-urocanic acid      | 0.1-10 |
|    | Cetostearyl alcohol    | 6. 75  |
|    | Propylene glycol       | 40     |
| 15 | Sodium lauryl sulphate | 0. 75  |
|    | Poloxamer 407          | 1      |
|    | Mineral oil            | 5      |
|    | Stringy petrolatum 12. | 5      |
|    | Buffering agent        | 0.01-1 |
| 20 | Purified water to      | 100    |

**Oinment Composition (% w/w)**

|    |                   |        |
|----|-------------------|--------|
|    | Cis-urocanic acid | 0.1-10 |
| 25 | Mineral oil       | 5      |
|    | Buffering agent   | 0.01-1 |
|    | Petrolatum to     | 100    |

It will be appreciated that the methods of the present invention can be incorporated  
 30 in the form of a variety of embodiments, only a few of which are disclosed herein. It  
 will be apparent for the expert skilled in the field that other embodiments exist and

do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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## CLAIMS

1. Use of a pharmaceutically acceptable agent or salt thereof being able to acidify the cell cytoplasm, for the manufacture of a pharmaceutical composition useful for causing immunosuppression in a person or an animal, wherein an effective amount of said agent is administered in an essentially non-dissociated form to the person or animal, and wherein the agent is admixed with a carrier adjusting the pH of the composition to the pH range 6.1 to 7.0.
- 10 2. The use according to claim 1 wherein the agent is an acid having its dissociation constant in the range 6.7 to 7.4, preferably in the range 6.9 to 7.3; most preferably about 7.0.
- 15 3. The use according to claim 1 or 2, wherein said agent is cis-urocanic acid and wherein the carrier is able to adjust the pH of the composition to 6.5 to 7.0.
4. The use according to any of the claims 1 to 3 for treatment or prevention of a disease or disorder curable by immunosuppression.
- 20 5. The use according to claim 4 wherein the disease or disorder is a local or systemic inflammatory disease, an autoimmune disease or an allergic condition.
6. The use according to claim 5 wherein the disease or disorder is a local or systemic inflammatory reaction, most preferably an inflammatory reaction involving the activation of cells of innate immune system, such as, a contact hypersensitivity or delayed type hypersensitivity reactions, acute graft rejection, psoriasis, dermatitis, periodontitis, mastitis, or vasculitis.
- 25 7. The use according to any of the foregoing claims wherein the agent is administered systemically or locally, preferably locally, most preferably topically.

8. Pharmaceutical composition comprising as active agent a pharmaceutically acceptable agent or salt thereof being able to acidify the cell cytoplasm, in combination with a pharmaceutically acceptable carrier, which carrier essentially prevents the agent from dissociating at extracellular pH values and wherein the  
5 carrier is able to keep the pH of the composition in the range 6.1 to 7.0.
9. The composition according to claim 8 wherein said agent is an acid having its dissociation constant in the range 6.7 to 7.4, preferably in the range 6.9 to 7.3; most preferably about 7.0.  
10
10. The composition according to claim 8 or 9, wherein said agent is cis-uropanic acid and wherein the carrier is able to keep the pH of the composition in the range 6.5 to 7.0.

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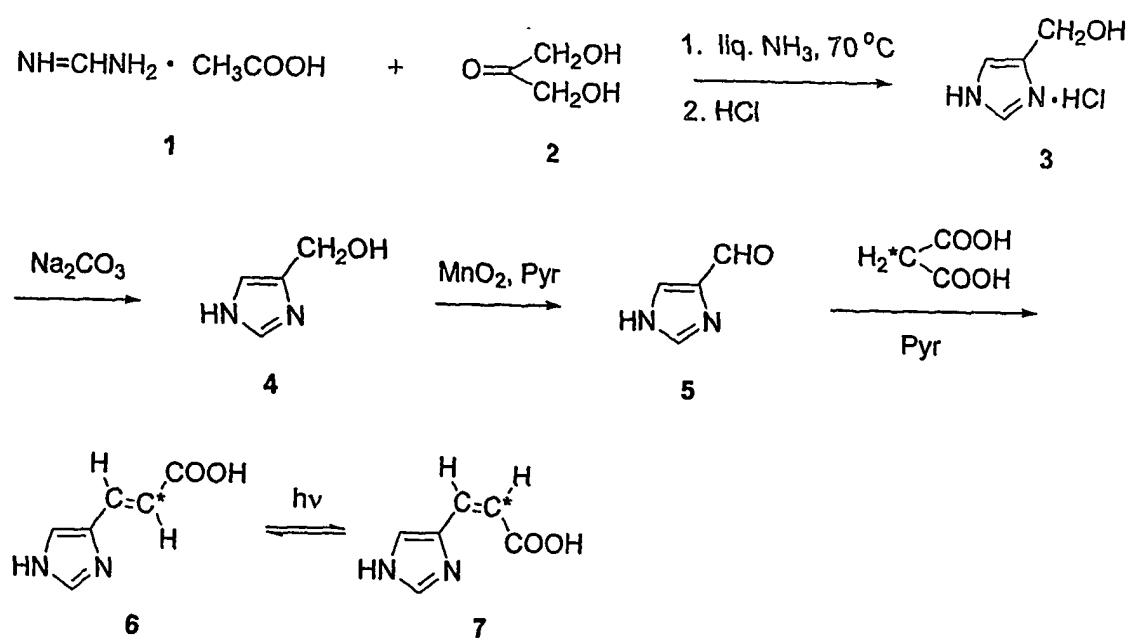


FIG. 1

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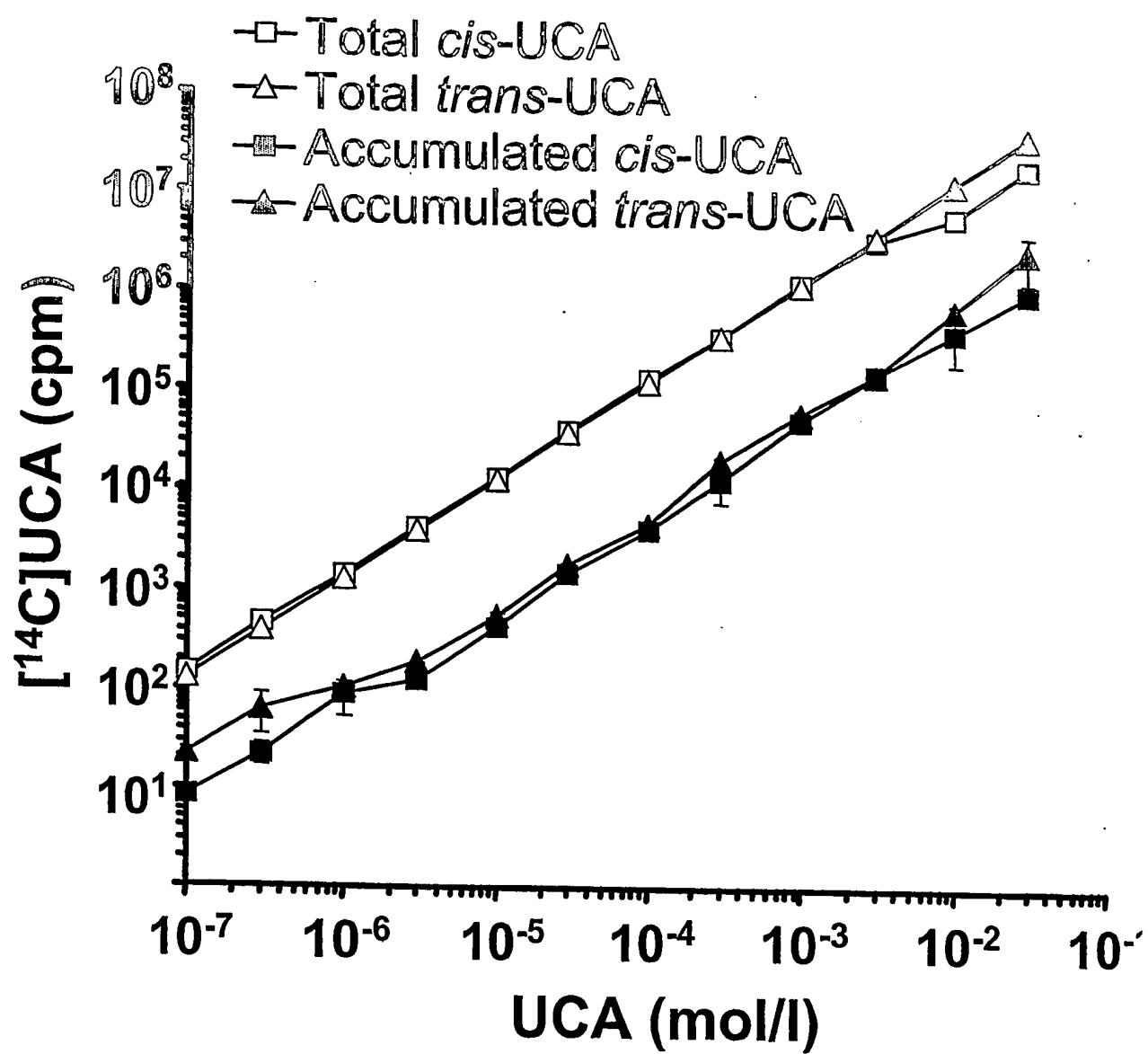


FIG. 2

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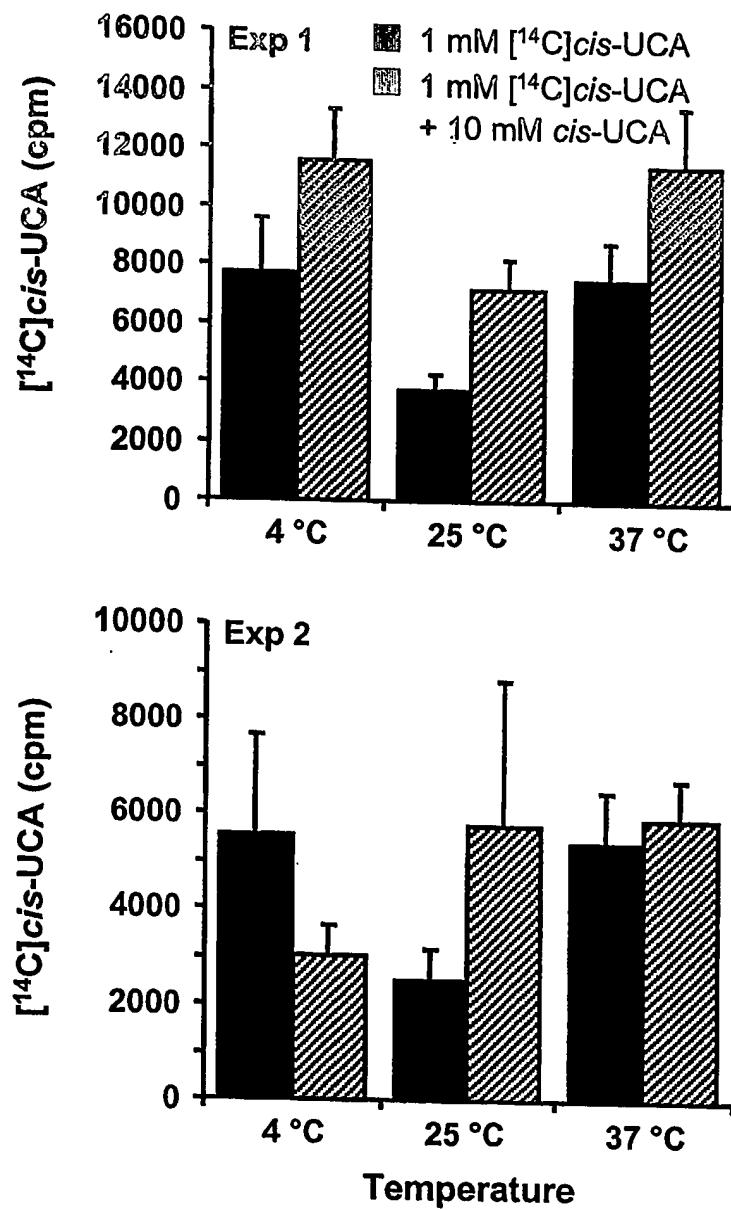


FIG. 3

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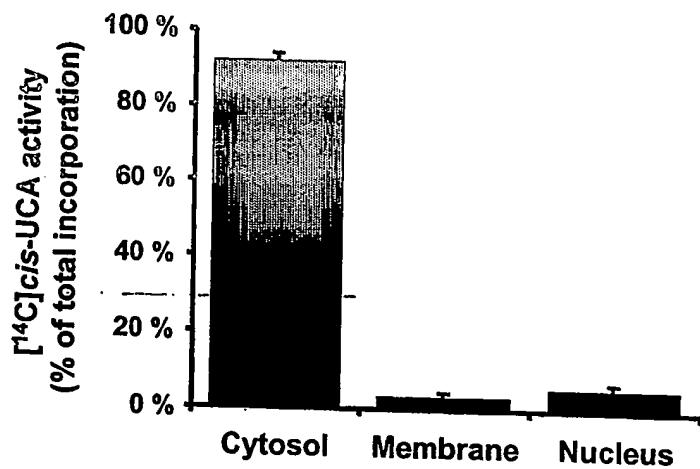


FIG. 4

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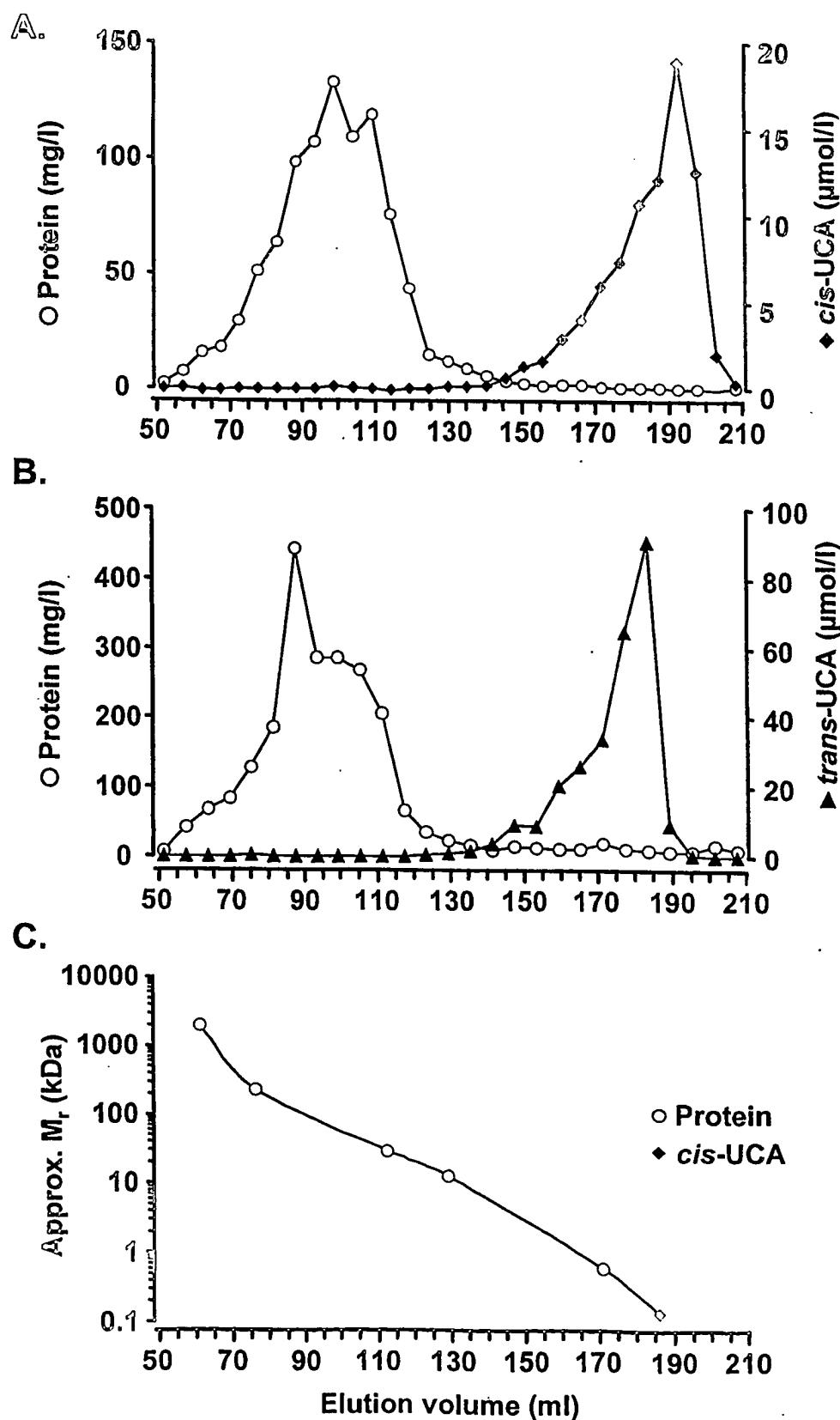


FIG. 5

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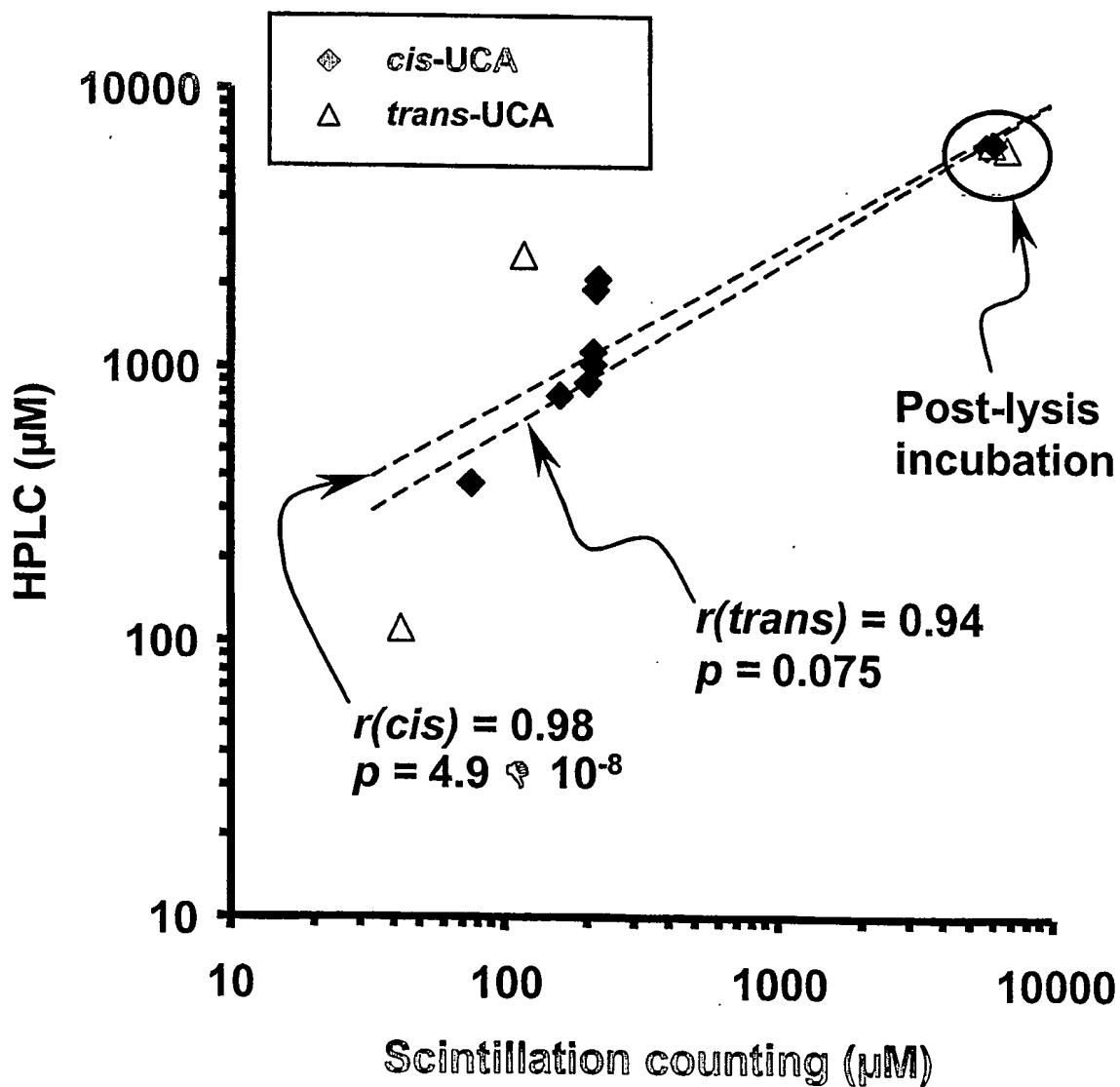


FIG. 6

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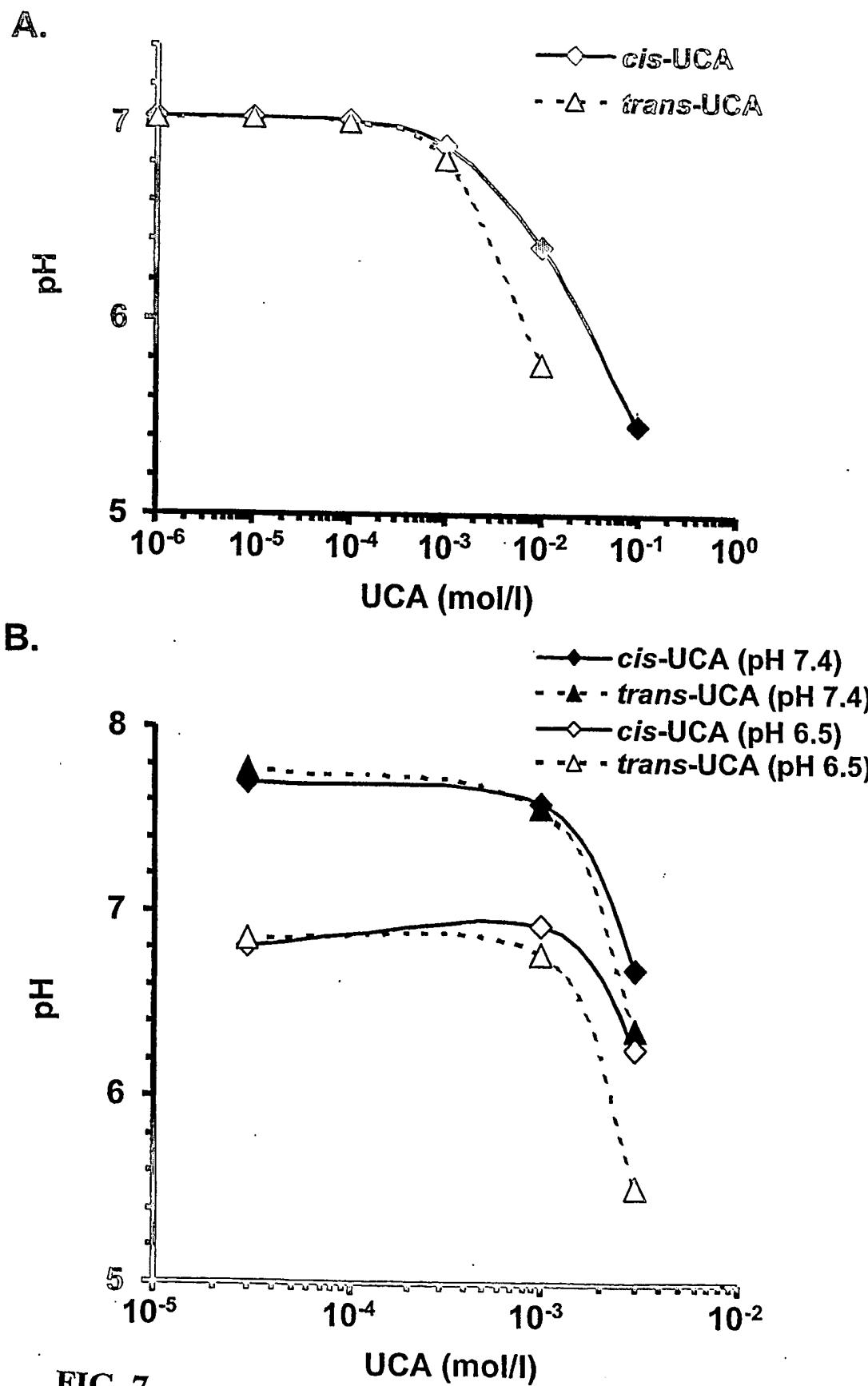


FIG. 7

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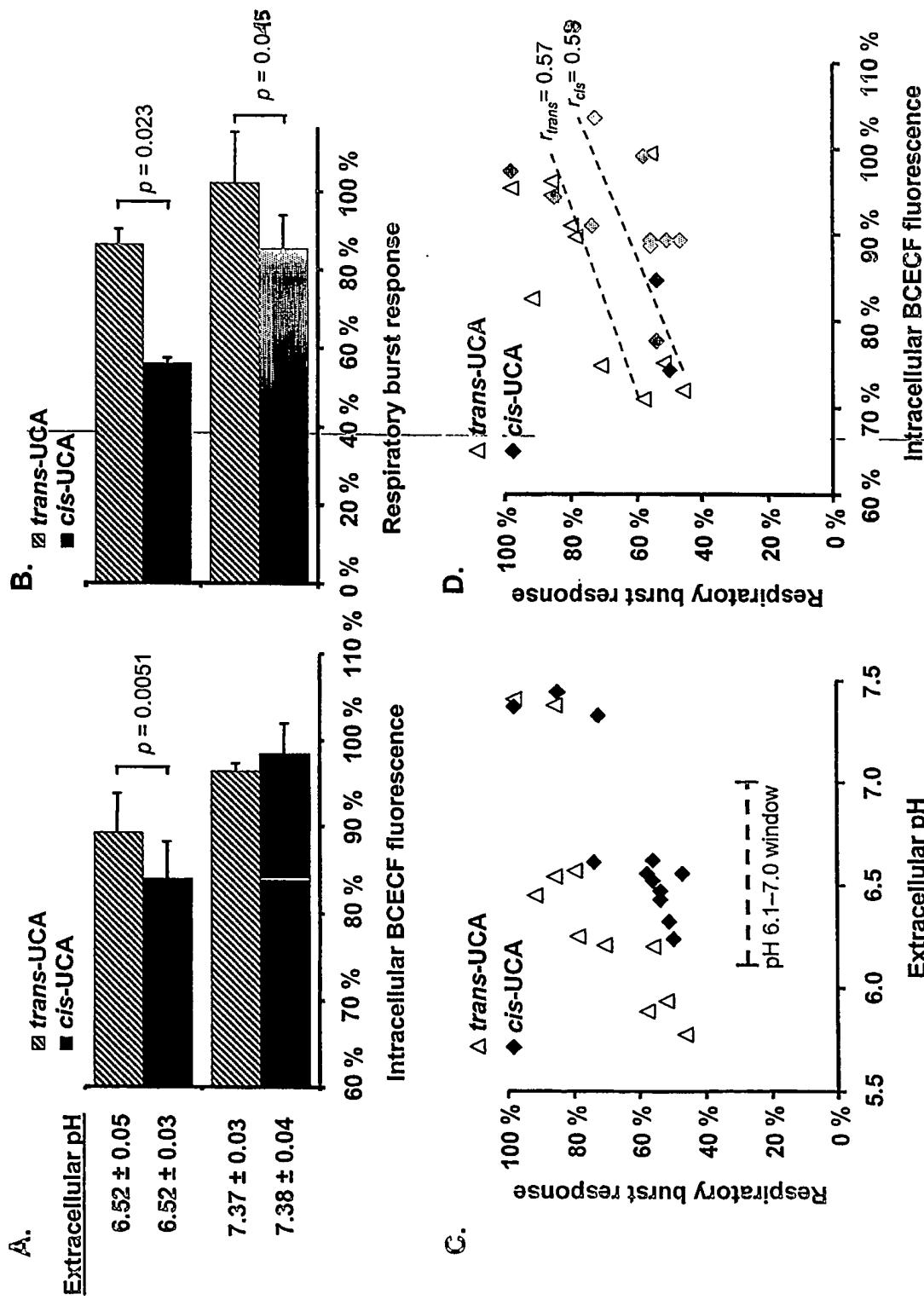


FIG. 8

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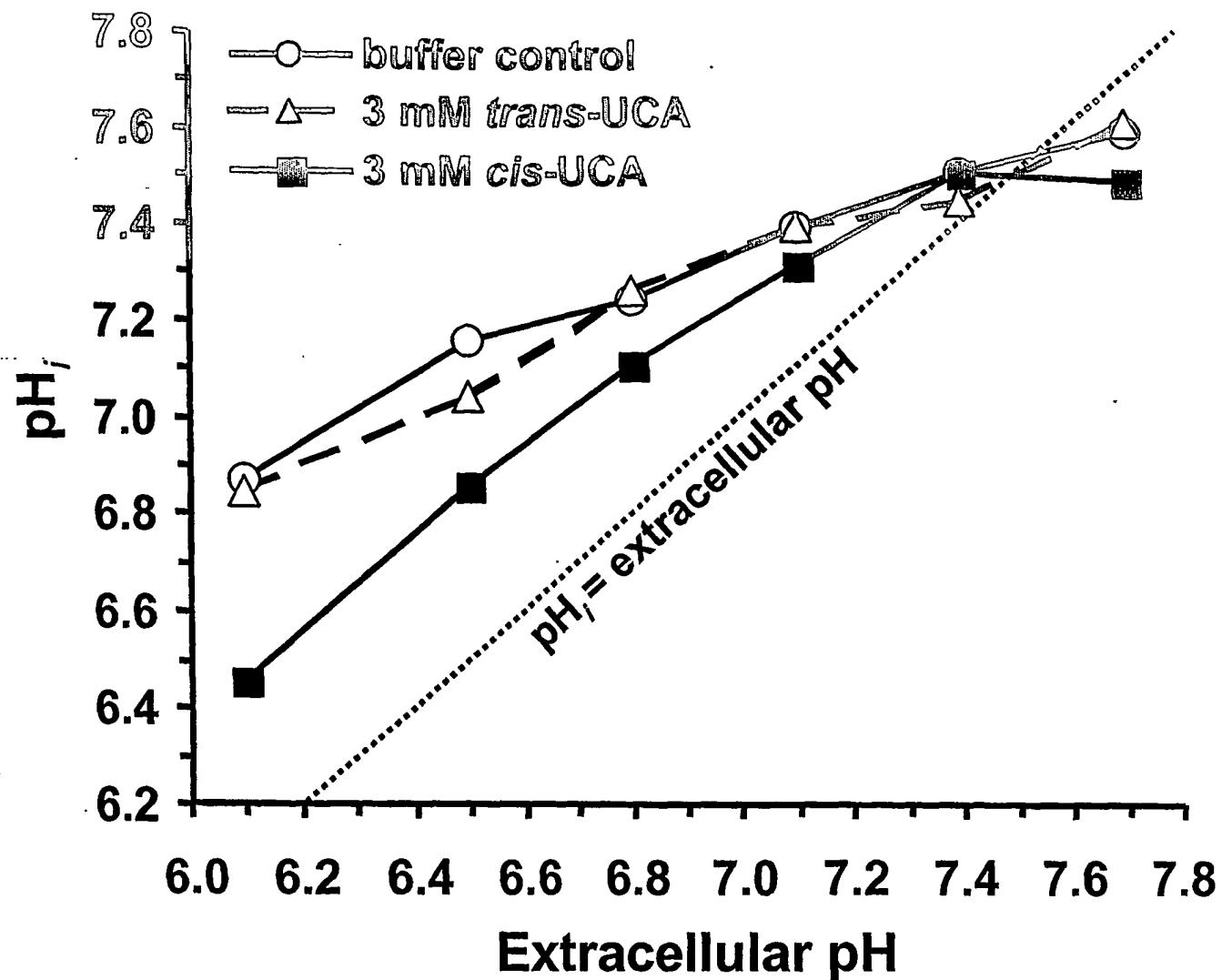


FIG. 9

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 2004/000109

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 31/415, A61K 31/185, A61P 37/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, MEDLINE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| X         | EP 0467116 A1 (BODE CHEMIE GMBH & CO.),<br>22 January 1992 (22.01.1992), page 3,<br>line 18 - line 21; page 9, line 3 - line 9,<br>and examples<br><br>-- | 1-10                  |
| X         | US 6028098 A (GOODMAN ET AL), 22 February 2000<br>(22.02.2000), example 1<br><br>--   | 1-10                  |
| X         | DE 4122497 A1 (OPTIMUM<br>PATENTVERWERTUNGSGESELLSCHAFT MBH),<br>21 January 1993 (21.01.1993), the claims<br><br>--                                       | 1-10                  |

 Further documents are listed in the continuation of Box C. See patent family annex.

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Date of the actual completion of the international search

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